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Structural Features of Methyl-Accepting Taxis Proteins Conserved between Archaebacteria and Eubacteria Revealed by Antigenic Cross-Reaction

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A number of eubacterial species contain methyl-accepting taxis proteins that are antigenically and thus structurally related to the well-characterized methyl-accepting chemotaxis proteins of *Escherichia coli*. Recent studies of the archaebacterium *Halobacterium halobium* have characterized methyl-accepting taxis proteins that in some ways resemble and in other ways differ from the analogous eubacterial proteins. We used immunoblotting with antisera raised to *E. coli* transducers to probe shared structural features of methyl-accepting proteins from archaebacteria and eubacteria and found substantial antigenic relationships. This implies that the genes for the contemporary methyl-accepting proteins are related through an ancestral gene that existed before the divergence of archaebacteria and eubacteria. Analysis by immunoblot of mutants of *H. halobium* defective in taxis revealed that some strains were deficient in covalent modification of methyl-accepting proteins although the proteins themselves were present, while other strains appeared to be missing specific methyl-accepting proteins.

Methyl-accepting chemotaxis proteins are central components in the chemotactic system of *Escherichia coli* (for a recent review, see reference 11). These transmembrane proteins, often called transducers, function as receptors for specific ligands and play a crucial role in sensory adaptation. Adaptation is mediated by changes in the extent of methylation at several specific glutamyl residues in the transducers, hence the name methyl-accepting chemotaxis protein. In *E. coli*, four different methyl-accepting transducers share structural and functional features but recognize different ligands. Several lines of evidence, summarized in the review cited above, support a model (14) for the transmembrane disposition of these 60,000-Da proteins in which two transmembrane regions, one near the amino terminus and the other approximately 40% of the way along the primary sequence, divide the protein into two hydrophilic domains. The periplasmic domain contains sites for ligand recognition; in this domain, few residue positions in the aligned sequences of the four transducers contain identical amino acids. The cytoplasmic domain functions in sensory signalling by controlling the activity of a protein kinase (3) and contains the methyl-accepting glutamyls. Over 50% of the residue positions in the four aligned sequences of the cytoplasmic domain contain identical amino acids, and many other positions have a different amino acid in only one of the sequences (2, 4, 10, 14, 20). Thus, it is not surprising that antisera raised to one transducer protein recognize the other transducers, even when the immunogen is Trg, the most distant member of the transducer family (8). Methyl-accepting taxis proteins have been observed in a number of bacterial species besides *E. coli* and *Salmonella typhimurium* (1, 6, 7, 9, 12, 13, 21–23, 29). An earlier study in this laboratory demonstrated that anti-Trg serum recognized specifically methyl-accepting taxis proteins from *Bacillus subtilis* and *Spirochaeta aurantia*. These species are only distantly related to the enteric bacteria or to each other (30), and thus, conservation of structural features among their respective methyl-accepting taxis proteins implies that these contemporary receptor proteins are related through an ancestral protein that was present in an early evolutionary form of eubacteria.

Archaebacteria are one of three primary groups of living organisms, distinct from both eubacteria and eukaryotes (30). Some biochemical or structural features of archaebacteria are shared with eubacteria, and others are shared with eukaryotes. Shared features are likely to have very early evolutionary origins, before the divergence of these three primary forms of life. Recent work (1, 24, 26, 27) has provided substantial information about methyl-accepting taxis proteins from the archaebacterium *Halobacterium halobium*. Some features of the archaebacterial proteins correspond with those of the well-characterized methyl-accepting chemotaxis proteins of *E. coli*, but other features are quite different. We used antisera raised to *E. coli* transducers to investigate the possibility that methyl-accepting proteins from archaebacteria and eubacteria shared structural features and found substantial antigenic relationships. The extensive cross-reactivity made it possible to use immunoblots to characterize electrophoretic patterns of methyl-accepting taxis proteins independent of methylation and thus to obtain new information about the sensory system in this archaebacterial species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Flx15 is a derivative of *H. halobium* OD2, which lacks bacteriorhodopsin and halorhodopsin but contains the sensory rhodopsins and is the wild type for chemotaxis (25, 28). The Pho mutants used in this study are derivatives of Flx15 described by Sundberg et al. (27, 28). Highly motile derivatives of the strains were obtained by several rounds of harvesting cells from the

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perimeters of chemotactic rings (for chemotaxis-positive strains) or of random spreading (for chemotaxis-negative strains) formed after 4 to 5 days of incubation at 37°C on plates containing 0.25% agar and a growth medium with Oxoid peptone (18). Subsequently, motile cultures were grown in Oxoid peptone medium at 37°C with rotary shaking.

Labeling with [*methyl*-^{3}H]methionine. L-[*methyl*-^{3}H]methionine (75 to 80 Ci/mmol; Dupont, New England Nuclear) was treated under vacuum in a Speed Vac to remove 2-mercaptoethanol before use. Cells were grown to mid-log phase (approximately 3.5 days), harvested by centrifugation, washed three times in growth medium without the peptone but with 0.1% arginine, and suspended at 0.8×10^9 to 1.0×10^9 cells per ml in the same medium. Puromycin was added at a final concentration of 100 μg/ml. After 30 min at 37°C, [*methyl*-^{3}H]methionine was added to a final concentration of 0.6 μM. Reactions were terminated at 60 min by mixing 1 volume of the labeled cell suspension containing 10^8 cells with 9 volumes of ice-cold acetone. Protein was collected by centrifugation, washed with 50% ice-cold acetone, and dried before solubilization in electrophoresis sample buffer.

Electrophoresis, immunoblotting, and fluorography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially by the procedure of Laemmli (15), with modifications described by Randall and Hardy (19). Proteins were transferred to nitrocellulose membranes by electroblotting at 30 V, which created approximately 100 mA, for at least 12 h (overnight) in a buffer consisting of 20 mM Tris and 150 mM glycine, pH 8.3, containing 0.025% (wt/vol) SDS and 20% (vol/vol) methanol. The extent of transfer was assessed by staining the gel after transfer with Coomassie brilliant blue R or the nitrocellulose membrane with Ponceau S.

Nitrocellulose sheets containing electroblotted protein were incubated with constant agitation by a rocking table for 1 h at 35°C in 20 mM Tris–250 mM NaCl, pH 7.5, containing 95 g of powdered milk per liter (Tris-PM). The sheets were rinsed three times with deionized water and then treated with a 1:200 dilution of antitransducer serum (or preimmune serum) in Tris-PM at room temperature for 1 h with agitation as described above. The nitrocellulose membranes were washed free of unbound antibody with three applications of Tris-PM containing 0.05% (wt/vol) Tween 20 for 10 min each. A 1:2,000 dilution of horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G (IgG) in Tris-PM was applied, the sheets were agitated at room temperature for 1.5 h, and unbound antibody was removed by washing as described above. Positions of the complex of antigen, antitransducer antibody, and peroxidase-linked second antibody were identified by using the chromogen 4-chloro-1-naphthol or a chemiluminescence reagent. The 4-chloro-1-naphthol was applied to the nitrocellulose sheet as a 0.5-mg/ml solution in 20 mM Tris–250 mM NaCl, pH 7.5, containing ice-cold 20% (vol/vol) methanol and H_2O_2 (0.015%), and the reaction was allowed to proceed for 4 to 5 h at room temperature. Alternatively, freshly prepared chemiluminescence reagent (ECL; Amersham) was applied to the nitrocellulose sheet, the reacted sheet was shielded by plastic, and X-ray film was exposed to the sheet for 1 to several minutes. For analysis of radiolabeled bands on a nitrocellulose sheet previously analyzed by immunoblotting, the sheet was washed with deionized water, dried overnight at room temperature, and sprayed three times with En^{3}Hance (Dupont) spray, with a period for drying after each spraying. X-ray film was exposed to the treated sheets at −70°C.

RESULTS AND DISCUSSION

Antiserum raised to highly purified Trg protein, obtained as described by Burrows et al. (5), exhibited substantial specificity for transducer proteins among the array of proteins in *E. coli*. In immunoblots of whole-cell material, there was a strong reaction with protein produced from chromosomal copies of normal transducer genes under conditions in which reaction with any other cellular component was essentially undetectable (16). This was the case even for cells containing Trg as the sole transducer protein at its normal level of 100 to 200 molecules per cell. Like an earlier anti-Trg serum raised to gel-purified protein (8), the newer serum recognized the other transducers, Tsr and Tar, with a slight reduction in sensitivity but with substantial specificity. This antiserum thus could serve as a useful probe for conserved structural features of methyl-accepting transducer proteins.

Recognition of *H. halobium* proteins by antitransducer sera. We used the serum for immunoblotting to test for cross-reacting proteins in extracts of *H. halobium* that had been subjected to SDS-PAGE and transferred to a nitrocellulose sheet. Our 14% polyacrylamide gels displayed the entire size range of proteins from *H. halobium*. Among all these proteins, a specific group of bands in the region of 90 to 120 kDa reacted with the antitransducer serum raised to Trg. Neither these bands nor any others reacted with serum taken from the rabbit prior to immunization. Essentially the same group of bands was recognized by antiserum raised to the Tsr transducer, provided to us by F. W. Dahlquist (University of Oregon). Figure 1 demonstrates that the cross-reacting bands correspond to methyl-accepting species. Methyl-accepting proteins were radiolabeled specifically by providing [*methyl*-^{3}H]methionine to cells blocked for protein synthesis (1). The radiolabeled cells were processed for immunoblotting, and the complexes of antigen, antitransducer antibody, and peroxidase-linked anti-rabbit IgG were detected by chemiluminescence. Once patterns of antibody reaction were recorded on an appropriately exposed film, the nitrocellulose sheet was dried, sprayed with a solution that would enable fluorographic detection of the *methyl*-^{3}H groups, and analyzed by fluorography. The two films, one with a record of antibody reaction and the other with the pattern of *methyl*-^{3}H-labeled proteins, could be aligned precisely with the nitrocellulose sheet and thus with each other. In Fig. 1, prints of those films are placed next to each other to reflect the alignment of the patterns, illustrating that the cross-reacting bands correspond to methyl-accepting species.

Specificity of recognition. We used preabsorption of antiserum with Trg protein as a control to assure that reaction with the archaebacterial proteins reflected recognition of antigenic determinants present on eubacterial methyl-accepting proteins. The antiserum was treated with membrane vesicles derived from cells lacking all transducer proteins or from otherwise isogenic cells containing high levels of Trg. Absorption with Trg-containing membranes eliminated reaction with *H. halobium* proteins, while absorption with membrane alone had little effect (Fig. 2). The patterns in Fig. 2 are the result of electrophoretic conditions different from those for Fig. 1, and thus, the arrays of methyl-accepting bands are more clearly resolved. Several strains with slightly different patterns are shown to document clearly the effects of preabsorption.

Examination of tactic mutants. A group of mutant strains of *H. halobium*, defective in chemotaxis and/or phototaxis, were recently characterized for a number of phenotypic

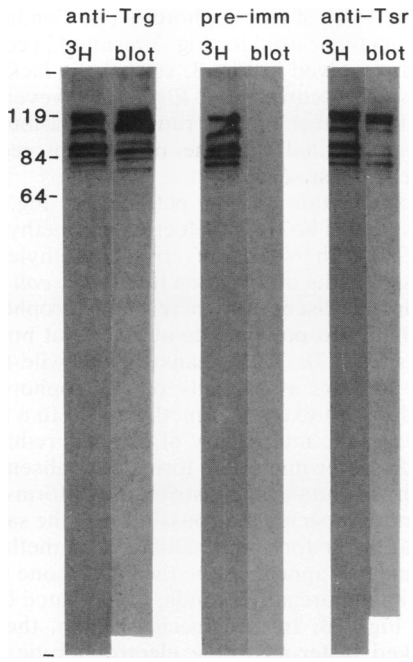

FIG. 1. Methyl-accepting proteins of *H. halobium* are recognized by antisera raised to transducer proteins of *E. coli*. Each pair of strips represents two analyses of the same lane of an SDS–14% polyacrylamide gel (0.37% bisacrylamide, pH 8.8) that had been electroblotted to a nitrocellulose sheet. The left lane (labeled "^{3}H") of each pair is a fluorograph of *methyl*-^{3}H-labeled material, and the right lane (labeled "blot") is an immunoblot with a particular rabbit serum, either an antiserum raised to Trg (anti-Trg) or Tsr (anti-Tsr) or serum taken from the rabbit prior to immunization with Trg (pre-imm). Addition of peroxidase-linked goat anti-rabbit IgG and the subsequent reaction with a chemiluminescence substrate produced the patterns shown. Each gel lane was loaded with an acetone precipitate from approximately 10^8 cells of the tactically wild-type strain Flx15 that had been labeled with [*methyl*-[^{3}H]methionine in the absence of protein synthesis. See Materials and Methods for details. The positions of the molecular size standards that are protein-dye complexes (prestained SDS-PAGE standard solution; Sigma Chemical Co.) are labeled (in kilodaltons): 119, β-galactosidase; 84, fructose-6-phosphate kinase; 64, pyruvate kinase. The top and the bottom of the separation gel are marked by dashes. Essentially the entire array of *H. halobium* proteins was displayed between these two extremes (see Fig. 1A in reference 1 for the range of molecular sizes included). Except for a weakly reacting band at approximately 70 kDa, not visible at these exposures, no other distinct bands were visible.

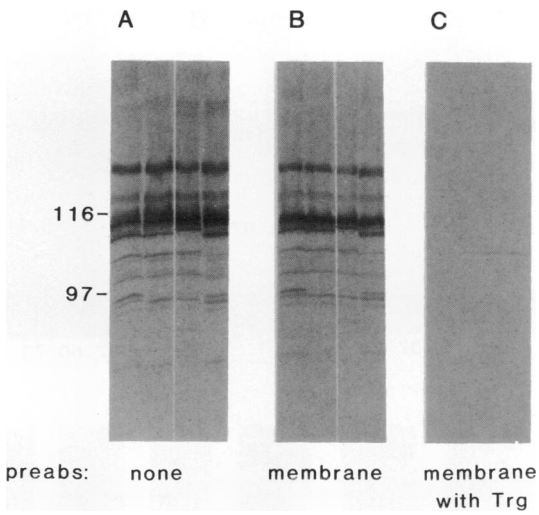

FIG. 2. Immunological cross-reactions of *H. halobium* proteins reflect antigenic determinants present on transducer proteins of *E. coli*. Samples of approximately 10^8 cells of Flx15, Pho5, Pho71, and Pho81 were run in triplicate on an SDS–10.5% polyacrylamide gel (0.07% biacrylamide, pH 8.1), electroblotted to a nitrocellulose sheet, and cut into three separate pieces corresponding to panels A, B, and C. Each piece was treated with anti-Trg serum (as described below), peroxidase-linked goat anti-rabbit IgG, and a chemiluminescence reagent to create the pattern on a film as shown in the figure. (A) Anti-Trg serum was used without any prior treatment. (B) Anti-Trg serum (100 μl) was preabsorbed (preabs) with membrane vesicles containing 475 μg of protein from *E. coli* CP553, which does not contain any transducer proteins, incubated for 1 h at room temperature, and centrifuged for 3 min at 14,000 rpm in an Eppendorf laboratory centrifuge. The supernatant was used to treat the nitrocellulose sheet. (C) Anti-Trg serum was treated the same as for panel B except that the membrane vesicles, containing 525 μg of protein, were from strain CP553 harboring pGB1, which carries the *trg* gene, and thus approximately 5% of the membrane protein was the Trg transducer. See the work of Burrows et al. (5) for descriptions of the vesicle preparation and the strains. Only the relevant portions of the gel lanes are shown. The positions of the molecular size standards indicated were determined by comparison to similar gels and are indicated (in kilodaltons) for β-galactosidase (116) and phosphorylase *b* (97).

properties, including electrophoretic patterns of *methyl*-^{3}H-labeled proteins (27). In that study, the methyl-accepting taxis proteins could be detected only as *methyl*-^{3}H-labeled species, a situation which limited the conclusions that could be made from the altered patterns. These limitations could be overcome by utilizing antitransducer antibodies to visualize methyl-accepting proteins in the absence of methylation. Thus, a set of mutants, representative of various phenotypic classes, was examined by immunoblotting with the anti-Trg serum (Fig. 3). The experimental procedure was like that described for Fig. 1 except that the electrophoretic conditions provided better resolution of the various bands of interest and antigen–antibody–second-antibody complexes were detected by a chromogenic substrate. The stained immunoblot was photographed before being processed for fluorography, and a print of the stained pattern was matched to the exact size of the nitrocellulose sheet and corresponding fluorograph. Figure 3A shows the fluorograph of *methyl*-^{3}H-labeled proteins from a wild-type parent and five representative mutant strains defective in phototaxis and/or chemotaxis; Fig. 3B shows the corresponding immunoblot of the nitrocellulose sheet from which the fluorograph was subsequently made. To facilitate comparison of *methyl*-^{3}H-labeled and immunoreactive species, the pairs of patterns for each strain were aligned in Fig. 3C. Parallel analysis with anti-Tsr serum produced patterns comparable to the ones in Fig. 3.

Mutants defective in covalent modification. Mutants such as Pho5, Pho60, and Pho71 that lack many *methyl*-^{3}H-labeled bands might exhibit that phenotype as the result of any one of three defects: absence of methyl-accepting proteins, absence of an active methyltransferase, or lack of an active methylesterase, resulting, respectively, in no substrate for methylation, no enzyme to catalyze the reaction, or no free methyl-accepting sites to be modified with a radioactive group. The data in Fig. 3 allow these possibilities to be distinguished and imply that Pho71 is defective in methyl-

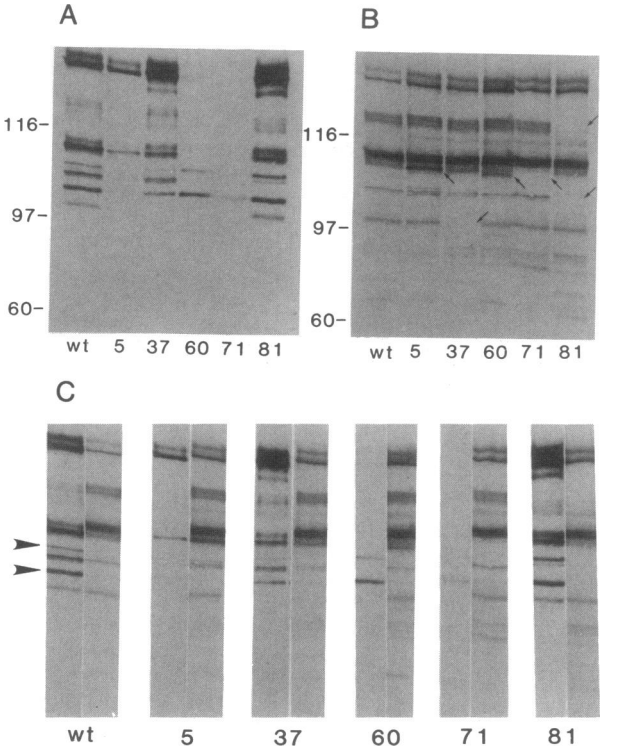

FIG. 3. Patterns of *methyl*-^{3}H-labeled and immunoreactive proteins from mutants defective in taxis. The experimental procedure was like that for Fig. 1 except that the gel conditions were altered (11% acrylamide and 0.073% bisacrylamide, pH 8.2) and the immunoreactive bands were detected by using 4-chloro-1-naphthol. (A) Fluorograph of *methyl*-^{3}H-labeled material that had been electroblotted to a nitrocellulose membrane; (B) immunoreactivity with anti-Trg of exactly the same nitrocellulose blot as in panel A; (C) patterns in the first two panels aligned in pairs corresponding to *methyl*-^{3}H label and immunoreactivity for each strain examined: Flx15 (lanes wt), Pho5 (lanes 5), Pho37 (lanes 37), Pho60 (lanes 60), Pho71 (lanes 71), and Pho81 (lanes 81). Only the relevant portions of the gel lanes are shown. The two higher-molecular-size standards are the same as for Fig. 2; the 60-kDa marker is α-amylase. Arrowheads in panel C indicate the pairs of bands that were not recognized by antitransducer serum.

transferase and that Pho5 and Pho60 are defective in methylesterase. The reasoning is as follows. Among the many *methyl*-^{3}H-labeled bands seen in patterns from wild-type cells, only two relatively faintly radiolabeled bands were visible in patterns from Pho60 or Pho71, and these two plus a few more were visible in patterns from Pho5. Figure 3 shows clearly that the pair of bands, marked by arrowheads in the margins of Fig. 3C, were not recognized by antitransducer serum. However, the normal array of immunoreactive bands was present in all the mutants but was not labeled or, in the case of Pho5, exhibited reduced labeling with radioactive methyl groups. Thus, these mutants are defective in the reactions of covalent modification but not in the production of methyl-accepting proteins, as predicted previously (27). In addition, the two methyl-accepting species marked by arrowheads in Fig. 3C appear unrelated to the other methyl-accepting proteins and may have functions different from those of the other methyl-labeled polypeptides (27). It should be noted that the relative electrophoretic positions of the methyl-accepting proteins are strongly influenced by the precise conditions of electrophoresis (1). Conditions slightly different from those used for Fig. 3 resulted in comigration of some bands resolved in Fig. 3, so that the lack of immunoreactivity was obscured (e.g., Fig. 1). However, analysis of a substantial number of gels run under various conditions clearly demonstrated that the pair is not recognized by anti-Trg or anti-Tsr serum.

Close examination of the patterns in Fig. 3 provides indications that Pho71 is defective in methyltransferase, while Pho5 and Pho60 are defective in methylesterase. The logic is based on the observation that for *E. coli* transducers, methylation can discernibly increase electrophoretic mobility of the modified polypeptide at some but not all methyl-accepting sites (17). This means that in wild-type cells, a transducer appears as a family of electrophoretic species, reflecting different extents of methylation. In a methyltransferase mutant, the multiplicity of forms is reduced because methylated, faster-migrating forms are absent, and in a methylesterase mutant, the distribution of forms is shifted to faster-migrating species. It is possible that the same phenomenon would occur for some halobacterial methyl-accepting proteins, and this appears to be the case in one region of the pattern of immunoreactive bands, as indicated by ascending arrows in Fig. 3B. In this specific region, the pattern for Pho71 lacked faster-migrating electrophoretic species and the patterns of Pho5 and Pho60 were enriched for them. As would be expected for a specific methyltransferase mutant, Pho71 exhibited no labeling of immunoreactive methyl-accepting proteins (Fig. 3) and a low basal rate of release of volatile methyl groups that was unaffected by any sensory stimulus (27). Consistent with Pho5 and Pho60 having, respectively, a partial and an extensive defect in methylesterase, the mutants exhibited low-level (Pho5) or trace (Pho60) labeling of methyl-accepting taxis proteins (Fig. 3) and reduced (Pho5) or no (Pho60) changes in demethylation rates upon strong sensory stimulation (27).

Mutants lacking specific methyl-accepting proteins. The substantial number and wide range of apparent molecular weights of immunoreactive, *methyl*-^{3}H-labeled bands suggest that there are several different methyl-accepting taxis proteins in *H. halobium*, but the possibility of electrophoretic diversity generated by methylation (see above) means that each band does not necessarily represent a distinct protein. However, if a specific mutation eliminated production of a particular methyl-accepting protein, then an immunoblot would reveal loss of one band or a set of bands. The strains surveyed in Fig. 3 include two such examples, Pho37 and Pho81. Pho37 lacks one particular immunoreactive, methyl-accepting polypeptide (marked by a descending arrow in the appropriate lane of Fig. 3B). Thus, this band is a candidate for being a distinct methyl-accepting protein. Pho81 is missing two sets of immunoreactive, methyl-accepting bands (marked by descending arrows in the appropriate lane of Fig. 3B). The lack of *methyl*-^{3}H-labeled bands at the lower position has been noted previously (1, 24, 27). Figure 3B and C show that the polypeptide, not just the modification, is missing, as is also the case for the higher-molecular-weight bands (upper descending arrow). The substantial difference in apparent molecular weight between the two sets of missing bands in Pho81 implies that they are not forms of the same polypeptide chain, but rather that one protein is represented by the group of electrophoretic forms in the upper region and a second protein is represented by the doublet at the lower-molecular-weight position. These observations indicate a complex phenotype for the phototaxis-negative, chemotaxis-positive mutant Pho81, since the

strain has already been shown to lack active forms of sensory rhodopsins I and II (27) and now appears to be defective in the production of two distinct methyl-accepting proteins.

How many different methyl-accepting taxis proteins exist in *H. halobium*? On the basis of the data in Fig. 3, a reasonable estimate would be five. This estimate is reached by neglecting the pair of bands not recognized by antitransducer sera as well as the faintly cross-reacting bands below 97 kDa in Fig. 3B, which are likely to be proteolytic fragments of intact transducers, and by assuming that groups of closely spaced bands are modified forms of a single protein. The five different proteins would correspond to the band missing in Pho37, the two sets of bands missing in Pho81, the doublet band uppermost in the patterns, and the group of bands that exhibit apparent shifts in Pho5, Pho60, and Pho71.

An ancient origin for methyl-accepting taxis proteins. Observations described here indicate that the methyl-accepting taxis proteins of the archaebacterium *H. halobium* are antigenically related to the well-characterized methyl-accepting transducer proteins of the eubacterial species *E. coli*. The antigenic cross-reactivity is quite substantial. The intensity of reaction of the anti-Trg serum with *H. halobium* methyl-accepting proteins from 10^8 cells was roughly comparable to the intensity of reaction of the antiserum with *E. coli* proteins contained in approximately the same number of cells. In an extensive survey of eubacteria for immunoblot reactivity with anti-Trg, many species exhibited specific cross-reacting bands, but few (primarily species closely related to the enteric bacteria) showed a reaction as intense as that observed for *H. halobium* (16). Even if the cellular dosage of methyl-accepting proteins were higher in *H. halobium* than in *E. coli* or most other eubacterial species, as might be expected from the larger size of the archaebacterial species, the intensity of cross-reaction indicates an extensive array of antigenic determinants shared between the methyl-accepting proteins of *H. halobium* and *E. coli*. This strongly implies that the genes for these proteins in the two contemporary species are descendants of a common ancestral gene that was present in an organism that existed before the divergence of archaebacteria and eubacteria. It is interesting to speculate that the postulated common ancestral gene coded for a transmembrane receptor protein and thus functioned in the sensory system of a very early form of life.

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